Chemical Chaperones Protect Epidermolysis Bullosa Simplex Keratinocytes from Heat Stress–Induced Keratin Aggregation: Involvement of Heat Shock Proteins and MAP Kinases

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Epidermolysis bullosa simplex (EBS) is a blistering skin disease caused by mutations in keratin genes (*KRT5* or *KRT14*), with no existing therapies. Aggregates of misfolded mutant keratins are seen in cultured keratinocytes from severe EBS patients. In other protein-folding disorders, involvement of molecular chaperones and the ubiquitin–proteasome system may modify disease severity. In this study, the effects of heat stress on keratin aggregation in immortalized cells from two patients with EBS (*KRT5*) and a healthy control were examined with and without addition of various test compounds. Heat-induced (43 °C, 30 minutes) aggregates were observed in all cell lines, the amount of which correlated with the donor phenotype. In EBS cells pre-exposed to proteasome inhibitor, MG132, and p38-mitogen-activated protein kinase (MAPK) inhibitor, SB203580, the proportion of aggregate-positive cells *increased*, suggesting a role of proteasomes and phosphorylation in removing mutated keratin. In contrast, aggregates were *reduced* by pretreatment with two chemical chaperones, trimethylamine *N*-oxide (TMAO) and 4-phenylbutyrate (4-PBA). TMAO also modulated stress-induced p38/c-jun N-terminal kinase (JNK) activation and expression of heat shock protein (HSPA1A), the latter of which colocalized with phosphorylated keratin 5 in EBS cells. Taken together, our findings suggest therapeutic targets for EBS and other keratinopathies.

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INTRODUCTION

The keratin intermediate filament network plays an important role in physically reinforcing keratinocytes, explaining why mutations in several keratin genes lead to heritable skin fragility disorders (Coulombe *et al.*, 2009). The best studied example of an inherited keratin disorder is epidermolysis bullosa simplex (EBS), which is characterized by intraepidermal blistering, owing to mutations in the keratin 5 and 14 genes (*KRT5* and *KRT14*), which encode for the major keratins of the basal cell layer (Coulombe *et al.*, 2009).

Based on the clinical severity, recent reclassification distinguishes three EBS subtypes: (1) the mild form, localized EBS (EBS-loc), (2) the moderate form, other generalized EBS (EBS, gen-nonDM), and (3) the severe generalized form, including the Dowling–Meara subtype (EBS-DM) (Fine *et al.*, 2008). In the generalized forms, pathognomonic keratin aggregates of misfolded K5 and K14 proteins are seen in the cytoplasm of basal cells in tissue samples (Anton-Lamprecht and Schnyder, 1982) and in patient-derived primary keratinocytes (Kitajima *et al.*, 1989; Ishida-Yamamoto *et al.*, 1991; Chamcheu *et al.*, 2010). Conceptually, the keratin aggregates reflect an accumulation of misfolded protein that should normally be removed by proteasomes, but to what extent this system is overloaded or faulty in EBS is not known.

Primary keratinocytes derived from EBS patients have recently been used to study heat stress-induced keratin aggregation and the protective effects of chemical chaperones (Chamcheu *et al.*, 2010). Alas, primary keratinocytes possess a limited lifespan and heterogeneous behavior with

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Abbreviations: EBS, epidermolysis bullosa simplex; ERK1/2, extracellular signal-regulated protein kinase 1/2; Hsp, heat-shock protein; JNK, c-jun N-terminal kinase; KRT, *keratin gene; MAPK, mitogen-activated protein kinase; 4-PBA, sodium 4-phenylbutyrate; PD, population doubling; TMAO, trimethylamine* N*-oxide*

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increasing passage numbers, making them unsuitable for more extensive functional assays. We and others therefore established patient-derived immortalized cell lines as a reproducible EBS model to study keratin remodeling and functional abnormalities that might shed further light on the pathogenesis of EBS (D'Alessandro *et al.*, 2002; Morley *et al.*, 2003; Liovic *et al.*, 2008; Chamcheu *et al.*, 2009; Russell *et al.*, 2010). By this approach, it has recently been shown that pretreatment of EBS cells with the chemical chaperone trimethylamine *N*-oxide (TMAO) attenuates keratin aggregation via yet unexplored mechanisms (Lee *et al.*, 2008; Chamcheu *et al.*, 2009). Analogously, several other molecules with pharmacological and/or chaperone potential, e.g., sulforaphane and 4-phenylbutyrate (4-PBA), are also known to modify EBS and other protein folding disorders (Collins *et al.*, 1995; Rubenstein and Zeitlin, 1998; Kerns *et al.*, 2007; Pruliere-Escabasse *et al.*, 2007; Yam *et al.*, 2007). The mechanism of action of 4-PBA is not fully understood, but in addition to being a chemical chaperone it modulates gene expression (Chung *et al.*, 2004; Gondcaille *et al.*, 2005).

Other putative mechanisms of TMAO and 4-PBA action involve heat shock proteins (Hsps) and mitogen-activated protein kinase (MAPK) signaling (Rubenstein and Lyons, 2001; Yam *et al.*, 2007; Gong *et al.*, 2009). In epidermal cells, both HSPA1A (HSP70) and HSPC1 (HSP90) are constitutively expressed and inducible by heat stress (Jonak *et al.*, 2006), whereas MAPKs are involved in the regulation of diverse cell stress responses (D'Alessandro *et al.*, 2002; Liovic *et al.*, 2008; Russell *et al.*, 2010).

The goal of this study was to evaluate the effects of heat shock on the EBS cytoskeleton and to determine putative mechanisms of the protective action of TMAO and 4-PBA. Here, we established two new immortalized keratinocyte cell lines (EB11 and EB12) derived from a severely affected EBS-DM patient with K5_p.GluE475Gly mutation. Together with previously established wild-type cell lines (control) and mutant cell lines from a generalized EBS non-DM patient with K5_p.Val186Leu mutation (Chamcheu *et al.*, 2009), the cells were observed over time after heat stress with or without pretreatment with chemical chaperones or inhibitors of proteasome and MAPK activities. In both types of EBS cells, stress-induced keratin aggregation led to increased levels of Hsp transcripts and proteins, and phosphorylation of p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase 1/2 (ERK1/2). Pretreatment with chemical chaperones decreased the fraction of cells containing keratin aggregates and modulated Hsp expression and MAPK activation in EBS cells. The study expands our understanding about the molecular mechanisms involved in cytoprotection by chemical chaperones, suggesting previously unreported targets for the treatment of EBS and other keratinopathies.

RESULTS

Growth characteristics and keratin expression profiles of newly established cell lines, EB11 and EB12

Immortalized keratinocytes, maintained in serum-free (EB11) or serum-containing (EB12) media, were established from a previously described patient (EBS1) carrying a K5_p. Glu475Gly mutation (Chamcheu *et al.*, 2010). Both cell lines have now exceeded >160 population doublings (PDs), whereas sham-transduced cells ceased growing after 38 PDs (Supplementary Figure S1 online). Immunostaining of K5 and K14 showed typical filamentous networks in EB11 and EB12 (Figure 1a), reminiscent of those found in the patient's primary cells (Chamcheu *et al.*, 2010). Virtually no keratin aggregates were seen in EB11 and EB12 at resting state, i.e., similar to the previously described cell lines, EB21 and EB22, from a patient with less severe EBS due to a K5_p.Val186Leu mutation (Chamcheu *et al.*, 2009).

The proportion of stress-induced keratin aggregates correlates with the EBS phenotype

Heat stress (43 °C for 30 minutes) induced keratin aggregates to a greater extent in EB11 and EB12 (Figure 1b and c) than in EB21, EB22 and immortalized normal keratinocytes (NKc11 and NKc12) (Figure 1d). Also, the intracellular localization and structure of the aggregates differed between the four EBS cell lines. In EB11 and EB12, most of the cytoskeletal network was affected (Figure 1b and c), whereas in EB21 and EB22 cells, keratin aggregates were mostly confined to the cell periphery (see Chamcheu *et al.*, 2009). Generally speaking, aggregates were less frequent when cells were grown in serum-containing medium, which reinforces the desmosomal connections, when compared with serum-free conditions. Analogously, in a pilot experiment, differentiated EB11 cells (1.5 mM $CaCl_2$ for 48 hours) showed a reduced proportion of aggregate-containing cells after heat stress compared with proliferating cultures (17 vs. 31%, $P<0.041$).

Chemical chaperones significantly reduce heat-induced keratin aggregates

In an apparently dose-dependent manner, pretreatment with TMAO significantly reduced the percentage of keratin-aggregate$^+$ cells in heat-stressed EB11 and EB21 cells, with a maximal 10-fold reduction seen in EB21 cells (Table 1).

Preaddition of 4-PBA also markedly reduced the fraction of aggregate-containing cells, with 1 mM of this compound being approximately equipotent to 50 mM of TMAO (Table 1). A similar protective effect of TMAO and 4-PBA was observed in EB12 and EB22 grown in the presence of serum (data not shown).

As the effects of added test compounds are best monitored without interference from serum factors, we continued our studies with EB11, EB21, and NKc11, and focused our interest on the mechanism of action of TMAO, which is the most well-established chemical chaperone.

TMAO modulates heat-induced expression of HSPA1A (HSP70) and HSPC1 (HSP90)

The previous notion that mutant keratin aggregate formation is a dynamic process in cultured keratinocytes (Werner *et al.*, 2004) and our own observations that exogenous chemical chaperones suppress aggregate formation (this and other studies), prompted us to investigate whether the intrinsic expression of chaperones (i.e., Hsps) in EBS cells is altered in response to cytoskeletal fragility and rescue by TMAO.

Already under resting culture conditions, *HSPA1A* mRNA levels were 50 and 100% higher in EB21 and EB11 cells

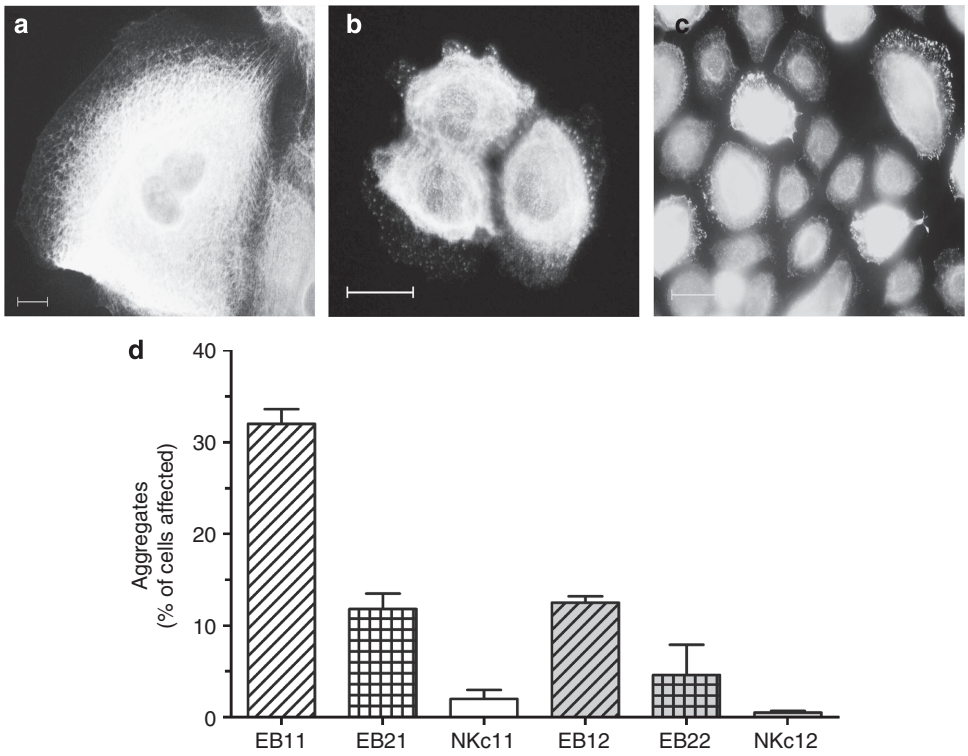


Figure 1. Heat stress induces keratin filament clumping in EBS1 cells to a higher degree than in EBS2 and healthy control cells. Immortalized cells from patient EBS1 were grown on glass cover slip, in serum-containing classic (EB12), or serum-free (EB11) medium, subsequently heat stressed for 30 minutes at 43 °C, allowed to recover for 15 minutes, and stained with an anti-keratin 5 antibody. (**a**) EB12 cells show a rope-like keratin network at resting state and (**b**) keratin aggregates in the outer cells of the colony when heat stressed, (**c**) whereas EB11 cells show keratin aggregates in most of the cytoskeletal network after heat stress. (**d**) The proportion of cells presenting keratin aggregates after heat stress of EBS1 (severe phenotype), EBS2 (mild phenotype), and control cells was counted in a minimum of 1,000 cells, showing a correlation with the phenotype as well as with the presence or absence of serum in the culture medium. Scale bars in **a–c** = 20 μm.

Table 1. Pretreatment with chemical chaperones reduced the formation of heat-induced keratin aggregates in EBS cells

		Cells presenting heat-induced keratin aggregates (%)		
Drug	**Concentration (mM)**	**EB11**	**EB21**	**NKc11**
—	0	32.0 ± 2.8	11.0 ± 1.4	2.0 ± 1.0
TMAO	50	8.7 ± 0.7***	3.6 ± 1.2*	0.7 ± 0.2
TMAO	100	5.3 ± 0.6***	1.0 ± 0.2*	0.4 ± 0.1*
—	0	25.9 ± 1.6	10.7 ± 0.8	ND
4-PBA	1	10.7 ± 1.7*	2.8 ± 0.7**	ND

Abbreviations: EBS, epidermolysis bullosa simplex (EB11, EB21); ND, not done; NKc11, control keratinocytes; 4-PBA, 4-phenylbutyrate; TMAO, trimethylamine *N*-oxide.
Cells were treated with TMAO or 4-PBA in serum-free conditions for 48 hours, heat stressed at 43 °C for 30 minutes, and stained with a K5 antibody after 15 minutes of recovery time. Keratin aggregates and total number of cells were semiquantitatively scored and results expressed as percentage of cells presenting keratin aggregates. A minimum of 1,000 cells were counted per cover slip in duplicate cover slips. The results represent the mean ± SD (n=3 for TMAO, n=2 for 4-PBA). Statistical analyses of untreated and treated cells were performed using analysis of variance (ANOVA; Bonferroni's multiple comparison test; *P<0.05, ***P<0.01, ***P<0.001).

compared with NKc11 cells (Figure 2a). After heat stress, *HSPA1A* expression increased, reaching a peak after 4 hours, which was of similar magnitude for all three cell lines. The mRNA levels returned to baseline within 24 hours, most rapidly so for EB11. Figure 2b shows the protein content of HSPA1A in the three cell lines, before and after heat shock, with and without TMAO pretreatment, respectively. In untreated cells, the expression of HSPA1A was highest in EB11 cells, which carry the most severe KRT5 mutation (Supplementary Figure S2b online). Upon heat stress without TMAO (Figure 2b, left panel), HSPA1A protein levels in EB11 and EB21 increased within 4 hours (Supplementary Figure S3 online), thus coinciding with the formation of keratin aggregates (see Figure 1).

Interestingly, TMAO exposure further increased the HSPA1A levels in unstressed cells (Figure 2b, compare –/– lanes in left and right panels), and these elevations were maintained after heat stress. In contrast, the mRNA and protein levels of HSPC1 (Hsp90) were only minimally affected by heat stress or TMAO, but were higher in EB11 and EB21 than in NKc11 cells at resting state (Figure 2c and d, left panel, and Supplementary Figure S4 online).

Taken together, the results suggest that pretreatment with TMAO attenuates the heat stress response of EBS cells *inter alia* by raising the HSPA1A level, which might reduce keratin aggregates under the assumption that both proteins colocalize in the keratinocytes.

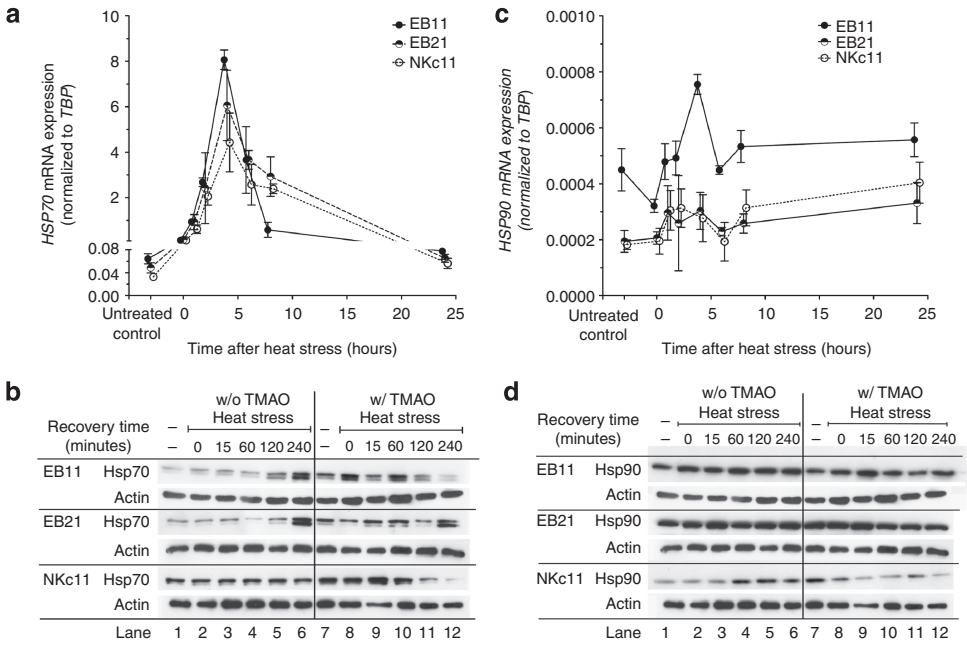


Figure 2. Effects of heat stress and trimethylamine *N*-oxide (TMAO) on mRNA and protein expression of HSPA1A (HSP70) and HSPC1 (HSP90) in epidermolysis bullosa simplex (EBS; EB11 and EB21) and control (NKc11) keratinocytes. The mRNA values of (**a**) *HSPA1A* and (**c**) *HSPC1* were monitored for 24 hours after heat stress at 43 °C for 30 minutes after normalization to the expression of the reference gene *TBP* ($n = 3$). The difference in HSP1A1 expression between EB11 and NKc11 at resting state was statistically significant ($P < 0.05$). The primers and probes are outlined in Supplementary Table S1 online. Protein expression was monitored in cell lysates (5 μg total protein) separated on SDS-acrylamide gel and blotted onto nitrocellulose membrane with antibodies against (**b**) HSPA1A or (**d**) HSPC1. β-Actin was used as loading control. Lanes 1 and 7 represent resting cells, whereas all other samples were taken 0–4 hours after heat stress. Lanes 7–12 represent cells preincubated for 48 hours with 100 mM TMAO.

Colocalization of Hsp70 (HSPA1A) and phosphorylated K5

Using *in situ* proximity ligation assay, a colocalization of phosphorylated K5 and HSPA1A was detected in untreated EB11 cells (Figure 3a) that increased upon heat stress (Figure 3b). In cells exposed to TMAO and 4-PBA, the colocalization signal increased further (Figure 3c and d, respectively).

These results support the hypothesized involvement of endogenous chaperons in the degradation of mutated keratin, a process that also implicates phosphorylation by MAP kinases known to be activated during cellular stress.

Heat stress and TMAO differentially modulate MAPK signaling

If TMAO affects MAPK, this could potentially explain its protective effects on keratin aggregation. We therefore monitored the activation of various MAP kinases (p38, JNK, and ERK) in relation to heat stress and TMAO treatment.

In conformity with the unaltered mRNA levels of p38-isoforms (*MAPK11-14*; data not shown), native p38 was highly expressed in all cell lines under resting condition, and heat stress did not affect the expression within 4 hours of recovery (Figure 4a, lanes 1–6). However, in TMAO-treated EBS cells, heat stress transiently *reduced* the levels of native p38, in contrast to NKc11 cells where the levels gradually increased (Figure 4a, lanes 8–12, and Supplementary Figure S5 online).

The levels of phospho-p38 in unstressed cells were higher in EB11 cells compared with EB21 and NKc21 cells (Supplementary Figure S2a and S2b online). Heat stress increased phospho-p38 levels between 0 and 60 minutes, which then

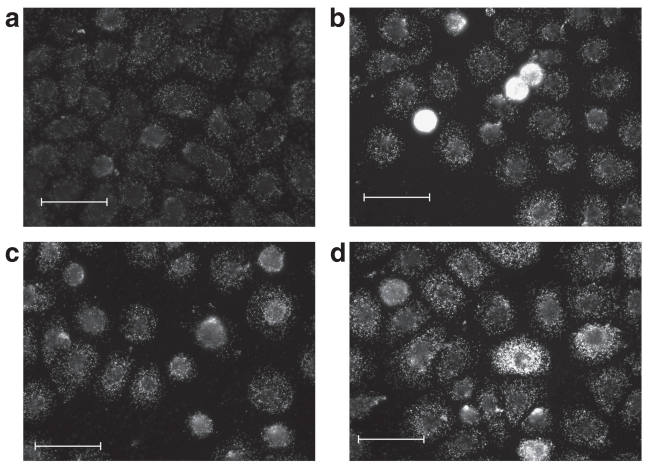
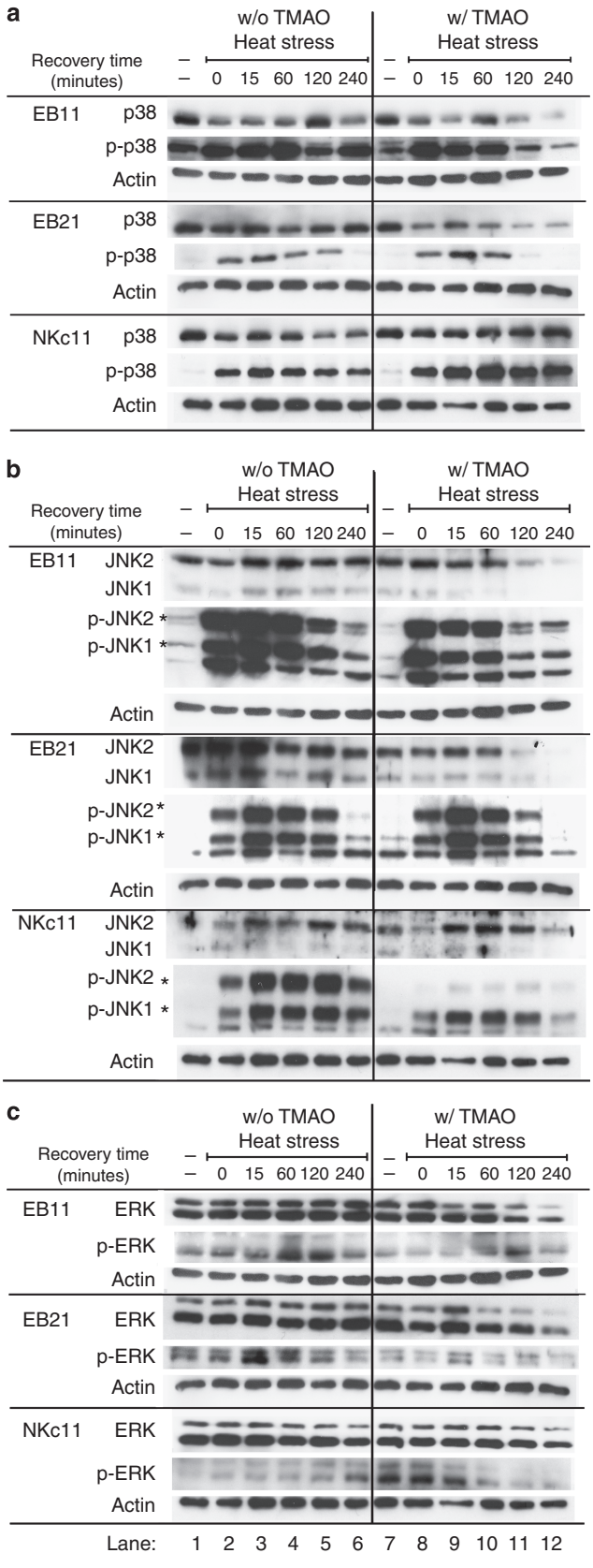


Figure 3. Phosphorylated K5 colocalizes with HSPA1A in heat-stressed EB11 cells. The proximity ligation assay (PLA) was used to study colocalization of phosphorylated K5 with HSPA1A (HSP70) in EB11 cells. A monoclonal antibody recognizing phosphorylated keratin 8 and 5 (clone LJ4; diluted 1:200) were used together with rabbit polyclonal HSP70 (Stress Marq; diluted 1:200) for *in situ* PLA (Söderberg *et al.*, 2006). Briefly, when the two secondary antibodies, with a DNA strand attached to each of them, are in close proximity (<40 nm), an added DNA template can be ligated to form a circle and initiate rolling circle amplification. (**a**) Unstressed cells, (**b**) heat-stressed cells (43 °C for 30 minutes), (**c**) heat-stressed cells pretreated with 100 mM trimethylamine *N*-oxide (TMAO), and (**d**) heat-stressed cells pretreated with 1 mM 4-phenylbutyrate (4-PBA). No signal was detected when omitting one of the primary antibodies or the PLA probes (not shown). Scale bars = 50 μm.



decreased until 4 hours in all cell lines. This effect was counteracted by TMAO treatment of the EB11 and EB21 cells at later time points (Figure 4a, lanes 7 and 8, and Supplementary Figure S5 online).

Native JNK1/2 protein levels at resting state were higher in EBS cells compared with NKc11 cells, irrespective of whether TMAO was added or not (Figure 4b and Supplementary Figure S6 online). Heat shock further increased the JNK levels in EBS cells, but this could be mitigated by TMAO pretreatment. Phospho-JNK1/2 was present already at resting state in severe mutant EB11 cells, and heat stress further induced phospho-JNK1/2 in all cell lines, especially in EB11 cells (Figure 4b and Supplementary Figure S6 online). In the presence of TMAO, both unstressed and heat-stressed cells showed reduced levels of phospho-JNK1/2 throughout the recovery time (Figure 4b, lanes 7–12, and Supplementary Figure S6 online).

Native ERK levels were similar in all cell lines and were not significantly affected by heat stress or TMAO when applied separately (Figure 4c and Supplementary Figure S7 online). However, in combination with heat stress, TMAO gradually reduced native ERK1 levels starting 1 hour after stress in all EBS cell lines (Figure 4c, lanes 8–12). The levels of phospho-ERK1/2 were higher in unstressed EBS cells than in control cells, and upon heat stress, a further transient increase occurred in EBS cells (Figure 4c and Supplementary Figure S7 online). In contrast, TMAO reduced the phospho-ERK1/2 levels in all EBS cell lines, which was contrary to its effect in NKc11 (Figure 4c, lanes 7–12).

In summary, MAPK signaling was altered in severely affected EB11 cells already under resting condition. After heat stress, both EB cell lines showed changes in phospho-p38, JNK1/2, and phospho-ERK activities that could be modulated by TMAO, thus coinciding with its ability to reduce the amounts of keratin aggregates. This prompted a further investigation of keratin aggregation in response to MAPK and proteasome inhibitors, which should theoretically enhance the accumulation of mutated protein.

Inhibition of MAP kinases and proteasome *increases* the proportion of keratin aggregate$^+$ cells

As MAPK is stimulated in the presence of keratin mutations and heat stress (see above), and the removal of keratin aggregates seems to involve proteasome-mediated degradation of phosphorylated protein (Löffek *et al.*, 2010), we added various MAPK and proteasome inhibitors to EB11 before monitoring the appearance of aggregate-containing cells.

Figure 4. The protein expression of mitogen-activated protein kinases (MAPKs) in heat-stressed epidermolysis bullosa simplex (EBS; EB11 and EB21) and control (NKc11) keratinocytes. Protein expressions of (**a**) p38 and phospho (p)-p38, (**b**) c-jun N-terminal kinase 1/2 (JNK1/2) and phospho (p)-JNK1/2, and (**c**) extracellular signal-regulated protein kinase 1/2 (ERK1/2) and phospho (p)-ERK1/2 were detected using western blot after applying 5 μg of total protein lysate. Lanes 1–6 represent heat-treated cells (43 °C for 30 minutes), and lanes 7–12 represent cells that were also preincubated for 48 hours with 100 mM TMAO. β-Actin was used as loading control. *Correct protein targets for p-JNK1/2 (bands below are unspecific binding of the antibody to p-p44/42).

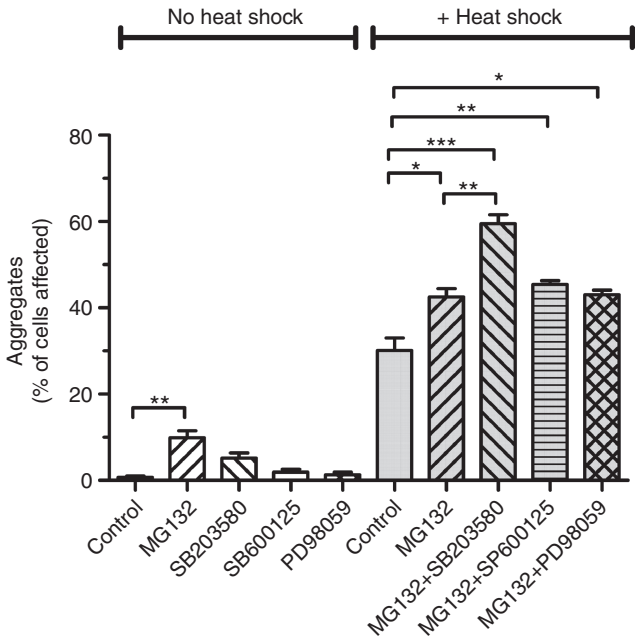


Figure 5. Keratin aggregation is enhanced by preincubation with proteasome and p-38 mitogen-activated protein kinase (MAPK) inhibitors. EB11 cells were preincubated for 4 hours with 500 nM MG132 (proteasome inhibitor), 10 μM SP600125 (c-jun N-terminal kinase (JNK) inhibitor), 10 μM SB203850 (p38 inhibitor), and 10 μM PD98059 (extracellular signal-regulated protein kinase (ERK) inhibitor) alone or in combination, followed in some cases by heat stress at 43 °C for 30 minutes (right panel). Keratin aggregated and total number of cells were semiquantitatively scored and results expressed as percentage of cells with keratin aggregates. A minimum of 1,000 cells were counted per cover slip in duplicate cover slips. The results represent the mean ± SD ($n = 2$). Statistical analyses were performed using analysis of variance (ANOVA; Bonferroni's multiple comparison test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Even in the absence of heat stress, keratin aggregation was increased by inhibitors of proteasome degradation (MG132) and MAPK-activated protein kinase-2/p38 MAPK (SB203580), whereas inhibitors of JNK (S600125) and MAPK/ERK (PD98059) had only minimal effects (Figure 5, left panel). Keratin aggregation after heat stress was further enhanced by adding MG132, especially when combined with SB203580 (Figure 5, right panel).

Interestingly, pretreatment with TMAO overruled the inducing effect of MAPK inhibitor on keratin aggregation (Supplementary Figure S8 online), suggesting that modulation of MAPK activity is incriminated in TMAO rescue of heat-stressed EBS cells.

DISCUSSION

EBS is mostly because of dominant mutations affecting KRT5 and KRT14, which compromise the keratin cytoskeleton integrity and cause intracellular cytolysis. In cell culture models the mutant keratins form short-lived aggregates (Werner *et al.*, 2004), which are reduced by addition of synthetic chaperones supposed to stabilize the misfolded proteins (Lee *et al.*, 2008; Chamcheu *et al.*, 2009, 2010, 2011). Thus, EBS was recently proposed to be a protein misfolding disorder (Lee *et al.*, 2008; Chamcheu *et al.*, 2009), where the dynamic keratin aggregates are reminiscent of static aggregates in neurodegenerative disorders (Bence *et al.*, 2001; Muchowski and Wacker, 2005). Although the cause of EBS is rather well understood, effective therapies are still lacking. It is expected that *in vitro* cultures of immortalized EBS cells can serve as a tool in screening for previously unreported therapeutic options. Two such promising approaches include gene and pharmacological therapies, the latter of which is at the focus of this study.

Here, we used two newly established immortalized K5_p.Glu475Gly mutant EBS keratinocytes maintained in serum-free or serum-containing medium (EB11 and EB12), together with previously established K5_p.Val186Leu mutant (EB21 and EB22) and wild-type (NKc11 and NKc12) cell lines (Chamcheu *et al.*, 2009). In accordance with previous findings in primary cells (Chamcheu *et al.*, 2010), heat stress of immortalized EBS cells provoked keratin aggregation that correlates with the clinical severity. The fate of the aggregates is largely unknown, but the accumulation of aggregates in EB11 cells after exposure to proteasome and MAPK inhibitors is in line with the proposed involvement of the ubiquitin–proteasome pathway in the decomposition of keratin aggregates (Löffek *et al.*, 2010). In contrast, addition of the chemical chaperones TMAO and 4-PBA drastically reduced the amounts of keratin aggregates in heat-stressed EB cells, thus strengthening the concept of using these compounds as the therapy of EBS.

There are several putative explanations for the reduction of keratin aggregates in the presence of TMAO and 4-PBA; e.g., the mutant keratins are physically protected from aggregation or they are primed for degradation by the ubiquitin–proteasome pathway. This also raises the question of whether endogenous chaperones and/or MAPK signaling could be involved in modulating keratin aggregation and hence explain the protective effects of TMAO and 4-PBA observed in cells from both EBS (this study) and epidermolytic ichthyosis patients (Chamcheu *et al.*, 2011).

Interestingly, EB11 cells, and to some extent EB21 cells, already at resting state expressed high levels of HSPA1A and phosphorylated MAPKs. This probably reflects an increased stress burden because of mutated keratin, and is in agreement with previous reports on other EBS cell lines (D'Alessandro *et al.*, 2002; Liovic *et al.*, 2008; Russell *et al.*, 2010). A further proof is that TMAO tends to normalize changes in Hsp and MAPK levels in the EBS cell lines, both at normal culture conditions and after heat stress, which is in conformity with previous reports in other protein misfolding disorders (Yam *et al.*, 2007; Gong *et al.*, 2009).

Furthermore, our data suggest that the TMAO-induced increase in HSPA1A, which colocalized with phosphorylated K5, is possibly implicated in facilitating the chaperone-mediated degradation of mutated keratins. A recent report suggested that HSPA1A acts as a linker between ubiquitin ligase and mutated keratins, followed by degradation of the ubiquitinated keratin by the proteasome pathway (Löffek *et al.*, 2010). Moreover, studies in other disease models showed specific effects of chemical chaperones on protein trafficking in conjunction to alteration of HSPA1A expression (Wright *et al.*, 2004; Gong *et al.*, 2009).

TMAO did not only affect Hsps, but also activated and modified members of MAPK signaling pathways. For instance, TMAO decreased the phosphorylation of p38 and JNK in EBS cells, suggesting a critical protective effect of TMAO that involves MAPKs. Moreover, colocalization of keratin aggregates with phosphorylated p38 (Wöll *et al.*, 2007) could link the TMAO-induced reduction of keratin aggregates to not only HSPA1A, but also activation of phospho-p38. The increase in aggregate-positive cells in the presence of a p38 inhibitor in EB11 cells is supporting a recent study suggesting that phosphorylation of keratins is necessary for degradation of the aggregates (Löffek *et al.*, 2010). It is also known from other cell types that stress-induced cell death correlates with the activation of JNK and ERK (Mosser *et al.*, 1997; Russell *et al.*, 2010). Thus, the effect of TMAO on phospho-JNK and phospho-ERK may protect EBS keratinocytes against apoptosis, although no direct effect on keratin aggregate formation was found. Taken together, the rescuing effect of TMAO on EBS cells seems to be more complex than the widely accepted hypothesis of a physical protection of the cytoskeleton, which however cannot be completely ruled out. At the same time, it should be emphasized that a reduction of heat-induced aggregates in EBS cells by TMAO and 4-PBA is not a proof of improved cytoskeletal stability of potential benefit to EBS patients, but could simply represent an epiphenomenon of no therapeutic value. This aspect needs to be further investigated in an animal model of EBS.

In conclusion, our overall results suggest that TMAO rescue of EBS cells is tightly linked to an activation of endogenous chaperones and proteasome-mediated degradation of keratins, which provides numerous previously unreported ideas for development of pharmacological therapies of keratinopathies.

MATERIALS AND METHODS

Patients and tissue samples

This study was approved by the regional ethical review board in Uppsala and was conducted with adherence to the Declaration of Helsinki Principles. Patients visiting the dermatology outpatient unit were recruited for this study after written and informed consent. Punch biopsies and primary keratinocytes were obtained from a generalized EBS-DM patient (EBS1), harboring a K5_p.Glu475Gly mutation (Chamcheu *et al.*, 2010).

Cell lines and culture conditions

Primary keratinocytes from patient EBS1 were immortalized in serum-free and classical serum-containing culture systems and were maintained and characterized as described elsewhere (Chamcheu *et al.*, 2009). This generated, to our knowledge, previously unreported EB11 and EB12 cell lines in serum-free and serum-containing medium, respectively. The keratinocyte cell lines, from patient EBS2 with mild EBS (EB21 and EB22) and from normal skin (NKc11, NKc12), were described earlier (Chamcheu *et al.*, 2009).

Heat stress assay and chemical chaperone treatment

Cells were subjected to heat stress as previously described (Chamcheu *et al.*, 2009). After heat stress, cells were allowed to recover, and thereafter harvested and subjected to analysis of keratin aggregate formation, and mRNA and protein expression, as described below. In other experiments, with chemical chaperones, cells were preincubated for 24–48 hours with nontoxic concentrations of TMAO (50 and 100 mM) and 4-PBA (1 mM) before heat stress and evaluation.

Immunocytochemistry, *in situ* proximity ligation assay, and microscopy

Cells grown on cover slips were fixed with 4% paraformaldehyde and immunostained for analysis of keratin aggregates as described elsewhere (Chamcheu *et al.*, 2009).

In situ proximity ligation assay was performed using the Duolink assay (Olink Bioscience, Uppsala, Sweden) essentially as described elsewhere (Söderberg *et al.*, 2006; Chamcheu *et al.*, 2011). Nonspecific binding sites were blocked followed by an overnight incubation at +4 °C with the monoclonal anti-pK5 (LJ4) (gift from Dr Omary) and the rabbit polyclonal HSP70/HSC70 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the sections were incubated with proximity ligation assay probe Minus and Plus, respectively, followed by hybridization, ligation, and amplification. Following detection, the sections were allowed to dry overnight at room temperature. After mounting in Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), the signal was detected using a Zeiss Axiophot microscope equipped with an Axiocam MRm camera and AxioVision Software (Carl Zeiss, Stockholm, Sweden).

RNA isolation, complementary DNA synthesis, and quantitative PCR

TRIzol Reagent (Invitrogen, Lidingö, Sweden) was added to the cultured cells and total RNA was isolated according to the manufacturer's instructions and first-strand complementary DNA synthesis was performed as described elsewhere (Törmä *et al.*, 2006). The complementary DNAs were stored at −70 °C and later the numbers of transcripts were determined by quantitative PCR. The 2 × TaqMan Universal PCR mix and Fast SYBR Green Master mix were used for probe- and SYBR-based detections on a 7500Fast cycler (Applied Biosystems, Stockholm, Sweden).

Preparation of cell extracts, SDS-PAGE, and western blotting

The cells were harvested for immunoblot exclusively as described elsewhere (Chamcheu, 2010; Forsberg and Rollman, 2010). Proteins were separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed with rabbit polyclonal anti-p38, anti-phospho-p38, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-ERK, anti-phospho-ERK (Cell Signaling, Danvers, MA), and mouse monoclonal anti-HSPA1A (HSP70) and anti-HSPC1 (HSP90) (Stress Marq, Victoria, Canada) diluted 1/1,000. It was visualized by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1/10,000), and using ECL Plus and ECL Hyperfilm (GE Healthcare, Uppsala, Sweden). Each film was scanned on a Flatbed Scanner (Epson, Hemel Hempstead, UK) and the intensities were quantified using ImageJ (NIH, Bethesda, MD).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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